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Tobacco cells contain a protein, immunologically related to the neutrophil small G protein Rac2 and involved in elicitor-induced oxidative burst

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Abstract Suspension-cultured cells of *Nicotiana tabacum* generated active oxygen species (AOS) when they were treated with the proteinaceous elicitor, cryptogein. This response was blocked by diphenylene iodonium, an inhibitor of the neutrophil NADPH oxidase. When microsomal extracts of tobacco cells were probed with an antibody directed against the human small G protein Rac2, two immunoreactive proteins were detected at 18.5 and 20.5 kDa. The same experiment performed with cytosolic extracts of tobacco cells led to the observation of a strong immunoreactive protein at 21.5 kDa only in the cryptogein-treated cells. The appearance of this cytosolic protein was related to the production of AOS by the elicited cells. These results provide evidence for the possible involvement of small G proteins, homologous to the neutrophil Rac2 protein, in the regulation of the elicitor-induced oxidative burst in plant.

Key words: Oxidative burst; Cryptogein; NADPH oxidase; Small G protein; Rac2; *Nicotiana tabacum*

1. Introduction

When plants are attacked by an avirulent strain of a pathogen, they exhibit a hypersensitive response (HR) which leads to rapid tissue necrosis at the sites of infection and limits pathogen growth to a small region of the plant [1]. A rapid and characteristic event of the elicited cells response is the release of active oxygen species (AOS) which is termed the oxidative burst [2,3]. Because the generation of AOS is believed to contribute to several resistance strategies of the plant [3,4], the mechanism of their formation and its regulation is a current topic of great interest [5].

This oxidative burst presents physiological similarities with the oxidative burst of the mammalian neutrophils: both are an early and transient response to invading organisms, and lead to a comparable rate of hydrogen peroxide production [2,6,7]. Therefore, it seemed interesting to investigate and determine whether the biochemical mechanisms of these two bursts were also similar. The neutrophil NADPH oxidase, responsible for $O_2^{\cdot-}$ production, is composed of several proteins: some located on the plasma membrane (p22 and gp91, respectively α and β subunits of the flavocytochrome b558) and others in the cytosol ($p47_{phox}$, $p67_{phox}$ and the small G proteins Rac1 and Rac2). Upon neutrophil activation, $p47_{phox}$, $p67_{phox}$, Rac1 and Rac2 move to the plasma membrane and associate themselves with the flavocytochrome, forming an electron-transport chain responsible for the reduction of molecular oxygen to $O_2^{\cdot-}$ [8,9]. In plants, proteins able to react with antibodies directed against human $p47_{phox}$, $p67_{phox}$, and p22 have already been found in soybean, *Arabidopsis thaliana* and cow pea cells [6,10,11], suggesting that biochemical similarities could indeed exist between animal and plant enzymes responsible for the oxidative burst. This has been confirmed by the recent cloning of a *gp91phox* homologue [12].

For the past few years, we have been studying the responses of *Nicotiana tabacum* cell suspensions to treatment with cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*. The earliest events detected are the binding to a specific high-affinity protein on the plasmalemma [13], the alkalization of the extracellular medium and concomitant electrolyte leakage [14], a fast and large influx of Ca^{2+} [15], and a transient production of AOS [16]. These responses are blocked by staurosporine, a known protein kinase inhibitor [16], giving evidence that phosphorylation steps are involved in transduction of the elicitation signal. However, nothing is known about the precise mechanism leading to AOS production. The presence of small G proteins, known to be potent modulators of the animal NADPH oxidase, have been demonstrated, mainly by molecular cloning, in various plant species [17,18] and their possible involvement in defense signal transduction pathways reported [7,19–21]. In this article we investigate the presence of proteins related to the small GTP-binding proteins Rac1 and Rac2 in tobacco cells and their modulation in microsomal and cytosolic fractions during the oxidative burst triggered by cryptogein.

2. Materials and methods

2.1. Materials

Tobacco (*N. tabacum* var. Xanthi) cell suspension cultures were grown in the medium of Chandler et al. [22] on a rotary shaker and used in the exponential growth phase.

Cryptogein was purified according to Ricci et al. [23], and used as an aqueous solution.

HL-60 cells obtained from American Type Culture Collection (Bio-Valley) were cultured in William's E-Ham's F10 medium (1/1, v/v) supplemented with 10% (v/v) fetal and 10% (v/v) newborn bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

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Abbreviations: AOS, active oxygen species; CL, chemiluminescence; DPI, diphenylene iodonium; HR, hypersensitive response; phox, phagocytic oxidase; RLU, relative luminescence unit; SOD, superoxide dismutase

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2.2. Determination of AOS production

Extracellular pH and AOS production were measured, at intervals, after addition of cryptogein to the medium of suspension-cultured tobacco cells. The production of AOS was determined by chemiluminescence using the luminol reagent and a luminometer (Lumat, LB 9501, Berthold): every 10 min a 250 µl aliquot of the medium was added to 50 µl of 0.3 mM luminol and 300 µl of assay buffer (175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 50 mM MES, pH 6.5). Values obtained at 10 s were reported.

2.3. Cell fractionation

As described above, changes in pH and AOS production were monitored in the culture medium of tobacco cells elicited or not with cryptogein. At different steps of AOS production, cells were collected by filtration and homogenized in grinding medium (50 mM Tris-MES pH 8.0, 500 mM sucrose, 20 mM EDTA, 10 mM DTT, 1 mM PMSF) using a Waring blender-homogenizer. The homogenate was centrifuged at 16000×*g* for 20 min. After centrifugation, the supernatants were collected, filtered through two successive screens (pore sizes 63 and 38 µm) and centrifuged at 96000×*g* for 35 min. The resulting membrane pellet was resuspended in storage buffer (10 mM Tris-MES, pH 7.3, 250 mM sucrose, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 20% glycerol) and stored at −80°C. Soluble proteins were precipitated overnight at 4°C by addition of saturated ammonium sulfate (45% final concentration) and centrifuged for 40 min at 120000×*g*. The pellet was washed with a washing buffer (10 mM Tris-MES, pH 7.3, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) centrifuged for 40 min at 120000×*g*, resuspended in storage buffer and stored at −80°C.

Protein content of membrane and cytosol preparations was measured according to Bradford [24] using bovine serum albumin as a standard.

2.4. Electrophoresis and protein immunoblotting

Samples were diluted 1:1 with Laemmli buffer and 30 µg proteins were loaded on a 14% SDS-polyacrylamide gel. After electrophoresis separation (2.5 h, 40 mA, Protean xi cell, BioRad), protein fractions were electroblotted onto nitrocellulose membrane (20 min, 15 V, Trans-Blot SD, semi-dry transfer cell, BioRad). Probing and detection of Western blots were performed as described in the ECL Western blotting detection kit (Amersham). Primary antibodies were used at 0.5 µg/ml in TBS-T (2 mM Tris, 15 mM NaCl, pH 7.6, Tween 20 0.05%). Antibodies (anti-Rac1 and anti-Rac2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxidase anti-rabbit Ig antibody (BioRad) was used at a 1:100000 dilution in TBS-T. The autoradiograms obtained after ECL revelation were analyzed with a Personal Densitometer (Molecular Dynamics).

3. Results

3.1. Effect of cryptogein on tobacco cells

The addition of 20 nM of the fungal elicitor cryptogein to tobacco cells led to a gradual and permanent alkalization of the culture medium (Fig. 1A) and to a transient production of AOS measured by chemiluminescence (Fig. 1B). Changes in pH always occurred earlier than AOS production, and AOS production became detectable at about pH 5.8.

Addition of DPI (10 µM), Tiron (1 mM), or catalase (100 µg/ml) to the cell suspension at the same time as cryptogein abolished the detection of AOS, unlike SOD (100 µg/ml) addition (Fig. 2).

3.2. Western blots

In order to determine if tobacco cells contain proteins similar to the small G proteins regulating the mammalian NADPH oxidase activity, different antibodies were used: (i) an affinity-purified polyclonal antibody raised against a peptide corresponding to the 14 amino acids mapping at the carboxy terminus of the human Rac1 protein, and (ii) an affinity-purified polyclonal antibody raised against a peptide mapping with 11 amino acids of the carboxy-terminal domain of human Rac2 protein. According to AOS production, four steps of the oxidative burst were considered (1–4, see legend of Fig. 1). Tobacco cells, elicited (T) or not (C) with cryptogein (20 nM), were fractionated at each of these steps, and the corresponding extracts were probed with the antibodies described above. Photographs of Western blots presented here are representative of at least four independent experiments.

When proteins extracted from the human myeloid HL-60 cells were probed with an anti-Rac1 antibody, a single strong immunoreactive protein was observed at 21.5 kDa, which is the reported molecular mass of the human Rac1 protein (Fig. 3). When the same experiment was performed with an equal amount of tobacco cell proteins, no significant signal was detected either in the microsomal or in the cytosolic extracts of plant cells (Fig. 3).

When proteins extracted from the human myeloid HL-60 cells were probed with an anti-Rac2 antibody, a strong immunoreactive protein was observed at 21.5 kDa and a lighter one at 20.5 kDa (Fig. 3). When cytosolic and microsomal extracts of tobacco cells were probed with the same antibody, strongly immunorevealed proteins were observed, the intensity

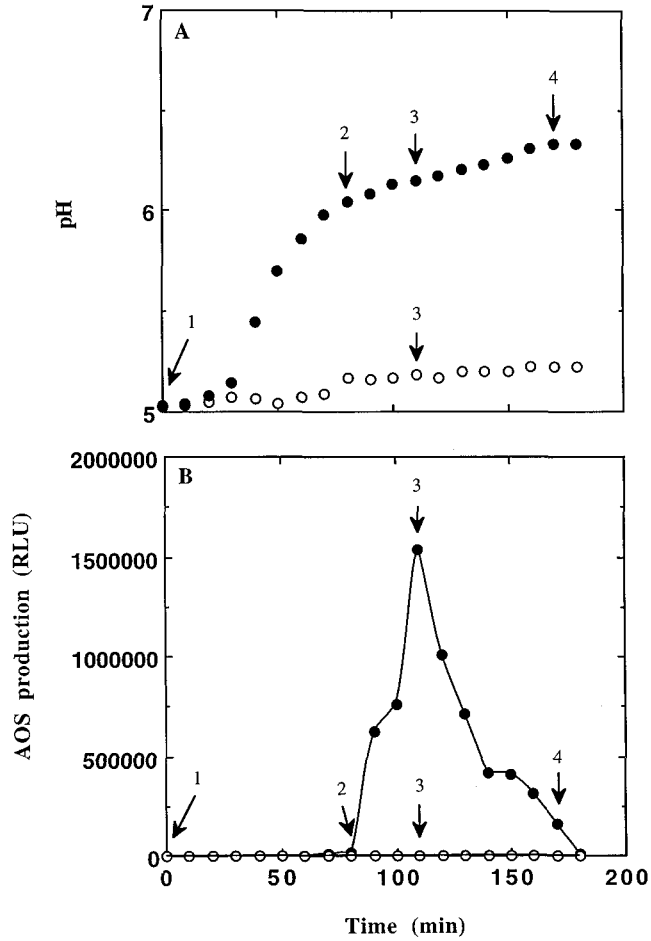

Fig. 1. Effect of cryptogein on extracellular pH and AOS production of by tobacco cells. At zero time of the experiment cryptogein 20 nM was added (●) or not (○) to the cell suspension. Every 10 min, extracellular pH changes (A) were monitored and AOS production (B) was determined by chemiluminescence as described in Section 2. The arrows indicate at what time of the elicitation process the different samples are harvested and subsequently analyzed with the Western blotting experiments described in Figs. 3–5.

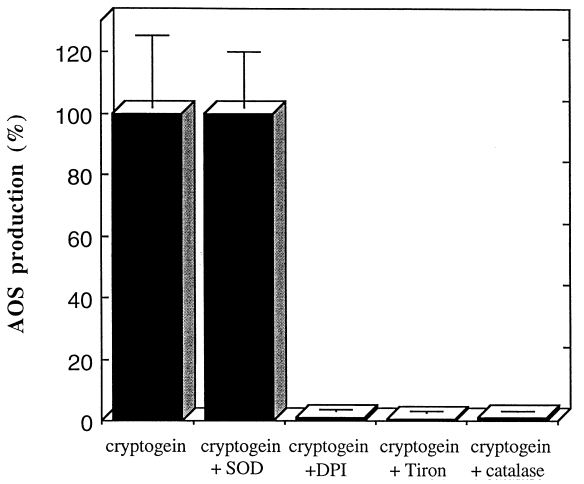

Fig. 2. Cryptogein-induced generation of AOS. Effect of SOD (100 μg/ml), DPI (10 μM), Tiron (1 mM) and catalase (100 μg/ml) on tobacco cells elicited with cryptogein (10 μM). Cryptogein and effectors were added at zero time. Data represent mean ± S.E. of three independent experiments. Values are presented as relative chemiluminescence with 100% representing the sum of CL values measured every 10 min during 180 min after the addition of cryptogein.

of which was different according to the samples. For plant microsomal extracts, two major immunoreactive proteins were observed at 18.5 ± 0.5 kDa and 20.5 ± 0.3 kDa and two very minor bands could also be detected at 19.4 ± 0.5 kDa and 21.4 ± 0.2 kDa (Fig. 3). No significant differences were observed between control and cryptogein-treated microsomal extracts. On the other hand, when plant cytosolic extracts were probed with the anti-Rac2 antibody, important differences were observed according to the samples. With cryptogein-treated samples harvested at maximal AOS production (T_3), a very strong signal was revealed at 21.5 ± 0.6 kDa, a lighter one at 18.5 ± 0.5 kDa, and an additional minor band could be detected at 19.3 ± 0.3 kDa (Fig. 3). For control samples (C_1, C_3) and for cryptogein-treated samples at zero time of elicitation (T_1), these immunorevealed proteins were present but hardly perceptible (Fig. 3). These observations were confirmed by a statistical analysis of four independent experiments performed with a densitometer (Fig. 4). A more detailed analysis of the 21.5 kDa immunoreactive protein in the cytosolic fraction during the elicitation process showed that its intensity increased from the beginning of AOS production, increased further during the oxidative burst and did not decrease when AOS production declined (Fig. 5).

4. Discussion

When the fungal elicitor cryptogein is added to tobacco cells, a rapid and permanent alkalization and a transient production of AOS are observed. The low pH of the medium of culture cells explains the lag period between cryptogein addition and AOS production: actually, in our model, the production of AOS is never detected unless the pH has reached 5.8. As the method used for detection of these AOS is luminol chemiluminescence, the main form observed is H_2O_2, as confirmed by the abolition of the luminol response when catalase is added to the assay. A nearly complete inhibition of this cryptogein-induced AOS production is observed when DPI (10 μM), an inhibitor of the neutrophil NADPH oxidase, or Tiron, a scavenger of $O_2^{\bullet-}$, was added to the cell suspension. This could suggest the existence of a membrane-located superoxide generating NADPH oxidase similar to the one which exists in the mammalian neutrophils, the primary AOS being $O_2^{\bullet-}$ which then dismutates to give rise to H_2O_2 detected in the chemiluminescence assay. This possibility has been reported by several groups on the basis of physiological evidence [25,26]. Moreover, recent reports indicate that proteins of different plant cells could react with antibodies directed against components of the neutrophils NADPH oxidase [6,10,11].

The use of an antibody directed against the human small G protein, Rac1, did not lead to a significant response either with microsomal or with cytosolic proteins of tobacco cells, whereas a strong signal was obtained with an extract of human myeloid HL-60 cells. In contrast, a very strong immunological response was observed when microsomal proteins from tobacco cells were revealed with an antibody directed against the human small G protein Rac2. The two major immunoreactive proteins (18.5 and 20.5 kDa) exhibited a molecular weight close to that usually described for this protein (21.5 kDa) and their intensity was quite similar to that obtained with an extract of human myeloid cells HL-60. Moreover, when the same antibody was used against cytosolic proteins of tobacco cells, a strongly immunoreactive protein of 21.5 kDa was only observed in the cryptogein-treated cells at their maximal rate of AOS production. Finally, the results described in Fig. 5 indicate that the detection of this 21.5 kDa protein corresponds to the beginning of the oxidative burst. However, this signal does not disappear when the AOS are no longer detected. These results, taken as a whole, indicate that a protein immunologically related to the neutrophil small G protein Rac2 is present in tobacco cells, and that its location inside the cell is modulated during the elicitor-induced oxidative burst.

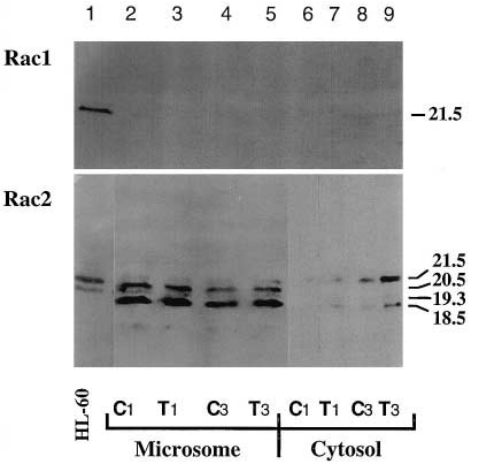

Fig. 3. Immunoblotting with antibodies directed to human neutrophils Rac1 or Rac2. Tobacco cells were treated (T) or not (C) with 20 nM cryptogein, harvested immediately (C_1, T_1) and at maximal rate (C_3, T_3) of AOS production, and fractionated as described in Section 2. HL-60 cells extracts (lane 1), tobacco microsomal extracts (lanes 2–5), or tobacco cytosolic extracts (lanes 6–9) were electrophoretically analyzed in a 14% polyacrylamide-SDS gel, transferred to nitrocellulose and probed with rabbit polyclonal antibodies against Rac1 or Rac2 (1/200 dilution). Horseradish peroxidase-conjugated goat anti-rabbit IgG (1/100 000 dilution) in combination with Enhancer Chemiluminescence reagents was then used to reveal polypeptides.

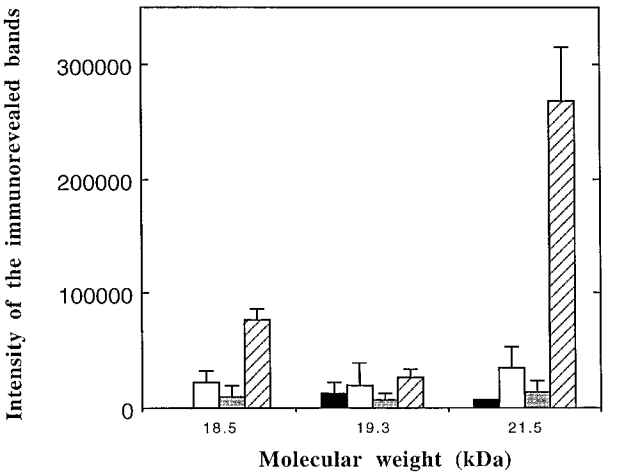

Fig. 4. Analysis of Western blots of tobacco cell cytosolic extracts probed with an anti-Rac2 antibody. Western blots corresponding to four independent experiments performed as described in the legend of Fig. 3 were analyzed with a Personal Densitometer (Molecular Dynamics). The intensities of all immunorevealed polypeptides of control samples C_1 (black), C_3 (white) or treated samples T_1 (gray), T_3 (hatched) are expressed in arbitrary units.

Rac1 and Rac2 present 92% amino acid identity and share GTP binding and GTP hydrolysis motifs with other members of the Ras superfamily. However, the antibodies used in these experiments, raised against a peptide mapping within the carboxy-terminal domain of human Rac1 or Rac2, are very specific for each of these proteins since no cross-reactivity is observed. This is confirmed by the absence of immunoreaction of tobacco cell proteins with the anti-Rac1 antibody, contrary to the result obtained with anti-Rac2 antibody. The search in data banks of sequences identical or very similar to the peptides used for antibody production did not identify any significantly homologous plant proteins. Moreover, the immunorevealed protein of tobacco cells exhibits similarities with the human Rac2 protein: an identical molecular weight, and the presence of apparent isoforms around 21 kDa that could correspond to the processed and unprocessed forms of the human protein [27]. Proteins of the Ras superfamily are, in animal cells, directed towards their regulatory targets in membranes by a series of post-translational modifications that include the attachment of a polyisoprenyl chain, the hydrolysis of three amino acyl residues and the methyl esterification of the carboxy-terminal prenylcysteine residue [28].

Both Rac1 and Rac2 are present in neutrophils [29,30] but Rac1 is expressed in a large number of different cell types whereas Rac2 is primarily expressed only in myeloid cells. Experiments carried out with a 'cell-free' system, i.e. neutrophil membrane and a crude or partially purified extract of a cytosolic fraction, demonstrated that Rac was needed for full oxidase activation [31]. The respective roles of these two small G proteins during the neutrophil activation process are still controversial, but it is generally accepted that, like the neutrophil oxidase components p40, p47 and p67, they are translocated from the cytosol to the plasma membrane to form an active electron transport chain and that a GDP/GTP exchange is involved in this activation process [29,30,32]. Moreover, the finding that $O_2^{\cdot-}$ production by GL1 cells was inhibited by Rac antisense oligonucleotides strongly supported the view that it represents an essential partner in the process of oxidase activation in vivo [33]. The immunoreaction of an anti-Rac2 antibody against tobacco cell proteins does not allow us yet to envisage its precise role in the cryptogein-induced oxidative burst. However, if the 21 kDa protein detected in tobacco cells is indeed homologous to the animal Rac2 protein, its mode of action could be somewhat different, since in resting neutrophils Rac is located in the cytosol and is translocated to the plasma membrane upon activation, whereas in tobacco cells the 21 kDa immunorevealed protein is present in the cytosol only after induction of the oxidative burst. The intense immunological response obtained with microsomal proteins will be properly interpreted only after subfractionation of the various cell membranes. Indeed, no significant variation of immunodetection is observed in this global fraction upon elicitation but this does not exclude that a variation of its amount in a particular component of rather low abundance occurs under such circumstances. Our current results do not allow us to choose between a putative translocation of Rac2-like protein from a cellular membrane to the cytosol and the hypothesis of its de novo synthesis.

Although small nucleotide binding proteins have been studied extensively in animal and microbial cells and a quite large number of small G proteins have been identified by molecular cloning from various plants [17,34], little information is available concerning the role of these proteins in plants. Indeed, most genes have been isolated on the basis of sequence homology with genes identified in other organisms and not on a functional basis, so the cellular functions of the corresponding proteins are still largely unknown. However, it has been demonstrated that expression of the gene encoding a small GTP-binding protein in transgenic tobacco abnormally induces salicylic acid in response to wounding and increases resistance to tobacco mosaic virus infection [19,35]. In other respects, evidence was given for participation of GTP-binding proteins in elicitation of the rapid oxidative burst in cultured soybean cells [7].

Finally, after the discovery of $p47_{phox}$, $p67_{phox}$ and $p22_{phox}$ [4], the demonstration in this paper of the presence of a Rac2

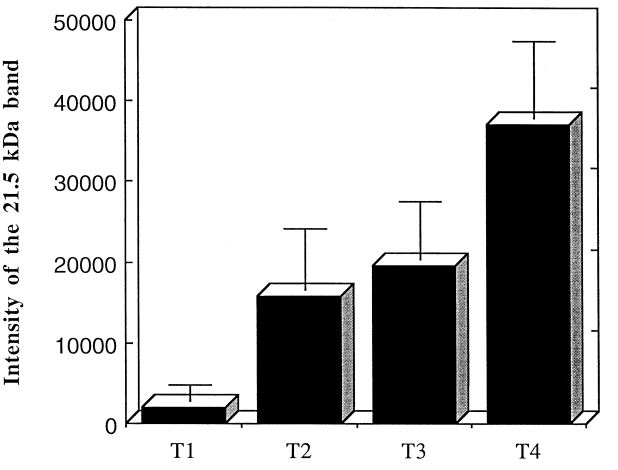

Fig. 5. Analysis of Western blots of tobacco cell cytosolic extracts probed with an anti-Rac2 antibody. Western blotting corresponding to four independent experiments performed as described in the legend of Fig. 3 were analyzed with a Personal Densitometer (Molecular Dynamics). The intensities of the 21.5 kDa immunodetected proteins of the treated samples T_1, T_2, T_3, T_4 are expressed in arbitrary units.

homologous protein in tobacco cells confirms the similarities between plant and animal oxidases. Moreover, the modulation of the location of this protein inside the cell upon elicitation provides new insights into the regulation of active oxygen species production. Further studies should allow us to purify the protein immunodetected in this work, in order to confirm its homology with the animal Rac2 G protein. Cloning the corresponding gene and expressing this component should provide a better understanding of the signal transduction leading to the oxidative burst in elicited plant cells.

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